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GB Virus C/Hepatitis G Virus Infection in Hemodialysis Patients: Determination of Seroprevalence by a Four-Antigen Recombinant Immunoblot Assay

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GB Virus C/Hepatitis G Virus (GBV-C/HGV) was identified recently and only two assays, consisting of a single recombinant protein, have been described for determination of the seroprevalence of this virus. An immunoblot assay was devised, which contains four recombinant GBV-C/HGV proteins. In this study, serum samples from 154 patients on maintenance hemodialysis were examined to assess the rate of seroreactivity against GBV-C/HGV. All sera were tested for the presence of antibodies by an in-house recombinant immunoblot assay, for GBV-C/HGV viremia by RT-PCR, and for HCV infection by PCR and by serological assays. Antibody reactivity against GBV-C/HGV was detected in 20.8% (n = 32) and viremia was found in 6.5% (n = 10) of the patients. In no case were viremia and GBV-C/HGV antibodies detected in parallel. HCV infection was observed in 15.6% (n = 24) by RT-PCR. In 20 of these patients, HCV antibodies were detected by enzyme immuno assay (EIA) and immunoblot assay. However, four of the HCV PCR-positive patients were negative by both serological tests. Only two patients were viremic for GBV-C/HGV and HCV in parallel. It is concluded that antibody reactivity against GBV-C/HGV is common among patients on maintenance hemodialysis. In contrast to HCV, parallel occurrence of GBV-C/HGV viremia and GBV-C/HGV seroreactivity was not observed. This suggests that GBV-C/HGV infection might be self-limiting. ***J. Med. Virol. 57:230–234, 1999.***

KEY WORDS: GBV-C/HGV; HCV; seroprevalence; immunoblot assay; PCR

INTRODUCTION

A new member of the family *Flaviviridae* was recently detected by molecular biological methods and named hepatitis G virus (HGV) [Linnen et al., 1996]. It shares sequence homologies of 99% to another recently detected virus, GB virus type C (GBV-C) [Simons et al., 1995; Leary et al., 1996]. Because of the very high sequence similarity, HGV and GBV-C are different isolates of the same virus. Therefore, they will be referred to as GBV-C/HGV. GBV-C/HGV is a single-stranded, enveloped RNA virus. Its genome, about 9,400 bases long, codes for structural proteins of the envelope (E1 and E2) and for nonstructural proteins (NS-2 to NS-5). GBV-C/HGV is phylogenetically closely related to hepatitis C virus (HCV) [Ohba et al., 1996]. Like HCV, GBV-C/HGV can be transmitted parenterally and vertically from mother to child [Feucht et al., 1996; Linnen et al., 1996]. However, the disease-inducing capacity of GBV-C/HGV in humans is still unclear.

Patients on maintenance hemodialysis are at increased risk for GBV-C/HGV viremia [Feucht et al., 1997b]. However, to determine the antibody prevalence only two serological assays, consisting of a single recombinant protein, have been described [Pilot-Matias et al., 1996; Tacke et al., 1997]. Therefore, we established a recombinant immunoblot assay to assess the seroprevalence of GBV-C/HGV in hemodialysis patients. It consists of four different recombinant proteins derived from the E1 region, the E2 region, the N-terminal part of NS3, and the C-terminal part of NS3 with a portion of the N-terminal NS4. Serum samples of 154 hemodialysis patients were screened by this assay to determine the prevalence of GBV-C/HGV seroreactivity. All samples were also tested for GBV-C/HGV viremia by RT-PCR and for HCV infection by RT-PCR and by serological assays.

MATERIALS AND METHODS

Patients

Serum samples from 154 patients undergoing chronic hemodialysis were examined by recombinant

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immunoblot assay and RT-PCR to determine the prevalence of GBV-C/HGV or HCV infection. A total of 62 women and 92 men were enrolled. Their ages ranged from 24 to 95 years (mean, 59 years). Apart from the history of hemodialysis, people investigated in this study had no other known risk factors for parenterally transmitted diseases. Informed consent was obtained from all participants.

Serological Assays

Nucleotide sequences of four different regions of the GBV-C/HGV genome—E1: amino acids (aa) 1–63; E2: aa 224–401; N-terminal part of NS3: aa 867–1273; C-terminal part of NS3 with N-terminal part of NS4: aa 1276–1531 (numbering of amino acid sequences as previously described [Linnen et al., 1996])—were amplified by RT-PCR and cloned into pTrxFus expression vector (Invitrogen, NV Leek, the Netherlands). To confirm the amplification products, parts of the nucleotide sequences were determined by the dideoxy chain terminating method using the modified T7 DNA polymerase (Sequenase version 2.0 kit; United States Biochemical, Cleveland, Ohio). After transformation into *Escherichia coli* GI724 (Invitrogen), expression was induced and GBV-C/HGV fusion proteins were isolated by affinity chromatography as recommended by the manufacturer. One μg of each soluble GBV-C/HGV fusion protein was suspended in 185 μl of 20-mmol/L Tris-HCl, pH 7.4, and 200-mmol/L NaCl, transferred to a separate slot of the blot apparatus and blotted on polyvinyl-difluoride (PVDF) membranes (Millipore, Eschborn, Germany). As an internal control, 1-μg and 375-ng immunoglobulin G (IgG) of a HCV- and GBV-C/HGV–negative standard serum (Behring, Marburg, Germany), and 1 μg of thioredoxin protein, were fixed onto the membrane. PVDF membranes were blocked using a 5% solution of skim milk (Oxoid, Basingstoke, England) by agitation on a rotation shaker for 30 min. The membrane was cut into 25 strips perpendicular to the slots of the blot apparatus.

The HCV recombinant immunoblot was established as previously described [Feucht et al., 1995]. Both immunoblot assays were performed as described [Feucht et al., 1995, 1997b]. Briefly, serum samples were diluted 1:100 in TBST (20-mM Tris-HCL, pH 7.4, 150-mM NaCl, and 0.05% Tween 20) and were exposed to blot strips for 1 hr on a rotation shaker at room temperature. The strips were then washed three times with 4-ml TBST for 5 min each and were exposed for 30 min at room temperature on a rotation shaker to alkaline phosphatase-labeled goat antihuman-IgG antibodies (Sigma, Deisenhofen, Germany), diluted 1:7,500 in TBST. Again, the strips were washed three times with 4-ml TBST for 5 min each and once with 4-ml TBS (TBST without Tween 20) for 5 min. Development was performed using a substrate solution (Western Blue, Promega, Heidelberg, Germany) containing BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) and Nitro Blue Tetrazolium. Both immunoblot assays were considered positive if at least antibodies against two different recombinant proteins were detectable. A second-generation HCV EIA (Abbott Laboratories, North Chicago, IL) was performed as recommended by the manufacturer.

PCR

HCV PCR was performed as previously described [Feucht et al., 1995]. In short, RNA was extracted by the guanidinium thiocyanate-phenol method [Chomczynski and Sacchi, 1987]. For cDNA synthesis, the isolated RNA was resuspended in diethylpyrocarbonate (DEPC)-treated H_2O, and 50 pmol primer no. 13 (5′-CCCAACACTACTCGCCTA-3′; nucleotide sequence 276–265 as previously described [Choo et al., 1991]), as well as 200-U reverse transcriptase (Life Technologies, Gaithersburg, MD) were added. After reverse transcription, amplification of the HCV cDNA was performed in a buffer containing 10-mM Tris-HCl (pH 8.3), 50-mM KCl, 2-mM $MgCl_2$, 160-μM (each) deoxynucleoside triphosphate, 30 pmol of each sense or antisense primer, and 2-U Pfu thermostabile DNA polymerase (Stratagene, La Jolla, CA). Primers no. 11 (5′-TTCGCGGCCGCACTCCACCATGAATCACTCCCC-3′; 1–17) and no. 12 (5′-AGTCTTGCGGCCGCAGCGC-CAAATC-3′; 255–244) were used. Amplification products were separated by agarose gel electrophoresis in 2% 3:1 NuSieve agarose (FMC, Rockland, ME), blotted onto positively charged nylon membranes (Qiagen, Hilden, Germany), and hybridized to a radioactively labeled probe.

GBV-C/HGV PCR was performed as previously described [Feucht et al., 1997b]. Briefly, RNA was extracted as described above. After reverse transcription with a cDNA primer from the NS-3 region of GBV-C/HGV (H4: 5′-CTCAAGCTTGAGAGCGCATCAGT-3′; 4472–4460 [Linnen et al., 1996]), amplifications were performed in a buffer of 10-mmol/l Tris-HCl (pH 8.3), 50-mmol/l KCl, 2-mmol/l MgCl, 160-μmol/l (each) deoxynucleotide triphosphate (dNTP) with 30 pmol of each sense (H1: 5′-CACGAATTCTATGGGCATGG-3′; 4278–4288) and antisense primer (H4), and 2 units of Amplitaq (Perkin Elmer Cetus, Emeryville, CA); denaturation for 30 sec at 94°C, annealing for 60 sec at 55°C, and extension for 60 sec at 72°C. In a second nested PCR, 30 pmol of each inner sense primer (H2: 5′-CTCGAATTCATGCGGACCGG-3′; 4305–4315) and inner antisense primer (H3: 5′-CTGAAGCTTC-CATCTTTGATGAT-3′; 4419–4406) were used. Amplification products were separated by agarose gel electrophoresis in 2% 3:1 NuSieve agarose (FMC), blotted onto positively charged nylon membranes (Qiagen), and hybridized to a radioactively labeled probe.

RESULTS

Sera from 154 patients on maintenance hemodialysis were examined for the presence of GBV-C/HGV and HCV antibody reactivity and for viremia. Antibodies against GBV-C/HGV were detected in 20.8% (n = 32) of the patients (Fig. 1). In 46.8% (n = 15) of the seroreactive samples, antibodies against all four recombi-

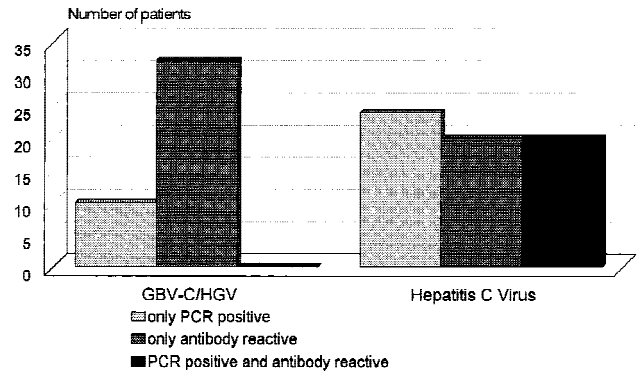

Fig. 1. Comparison of antibody reactivity and viremia in GBV-C/HGV- and HCV-infected hemodialysis patients.

nant proteins were observed, whereas in 6.2% (n = 2) reactivity was only directed against two proteins. Antibodies against the E2-derived recombinant protein were detected in 93.8% (n = 30), but two seropositive samples showed no reactivity against this protein. Immune response against the individual recombinant proteins was as follows. In 93.8% (n = 30), the antibodies were directed against the E2 recombinant protein; in 84.4% (n = 27), against the NS-4 protein; in 81,3% (n = 26), against the NS-3 protein; and in 78,1% (n = 25), against the E1 protein (Table I). None of the seroreactive patients had detectable GBV-C/HGV viremia by PCR. However, GBV-C/HGV RNA was detected in 8.2% (n = 10) of the seronegative patients (five females and five males, respectively). Neither the antibody-reactive nor PCR-positive patients had clinical or biochemical signs of liver disease except those who were also infected by HCV.

HCV infection was detected by RT-PCR in 15.6% (n = 24) of the dialysis patients. All samples were also tested by a second-generation HCV EIA and HCV recombinant immunoblot assay, and positive results were obtained in 83.3% (n = 20). Four of the PCR-positive samples displayed no antibody reactivity by either HCV-specific serological assay.

Two patients were positive for both HCV RNA and GBV-C/HGV RNA. One was a 54-year-old female, the other a 28-year-old male. Both had elevated liver enzymes (ALT values >30 IU/ml) at the time of investigation. An additional eight HCV-positive patients were also positive by GBV-C/HGV immunoblot assay (Table II). However, GBV-C/HGV RNA was not detected in any of them.

TABLE I. Distribution of Reactivity Patterns in the GBV-C/HGV Recombinant Immunoblot Assay Among the 32 Antibody-Positive Patients

Reactivity pattern of GBV-C/HGV immunoblot				Number of patients (n = 32) (%)
E1	E2	NS-3	NS-4	
+	+	+	+	15 (46.8)
−	+	+	+	5 (15.6)
+	+	+	−	4 (12.5)
+	+	−	+	4 (12.5)
+	+	−	−	1 (3.1)
+	−	+	+	1 (3.1)
−	+	−	+	1 (3.1)
−	−	+	+	1 (3.1)

TABLE II. Comparison of GBV-C/HGV Antibody-Reactive and Antibody-Negative Patients With Regard to Different Features

Feature	GBV-C/HGV antibodies		All
	Positive	Negative	
Sex (M/F)	19/13	73/49	92/62
Age (years), range/mean	26–95/62	24–90/58	24–95/61
GBV-C/HGV RNA-positive	0	10	10
HCV RNA-positive	8	16	24
GBV-C/HGV RNA- and HCV RNA-positive	0	2	2
HCV AK-positive	7	13	20

DISCUSSION

Sera from 154 patients on maintenance hemodialysis were screened for antibodies against GBV-C/HGV by an in-house immunoblot assay. Most patients seem to develop antibodies against the E2 region, since antibody reactivity was directed against this protein in our assay in more than 93% of the samples. However, 6.3% (n = 2) of the serologically reactive samples would have been missed by using the E2-derived protein alone.

In the present study, 20.8% of the hemodialysis patients were found to be GBV-C/HGV antibody-reactive. This prevalence does not vary significantly from the seroprevalence observed among healthy blood donors [Feucht et al., 1997b; Lou et al., 1997]. However, patients on maintenance hemodialysis are known to have impaired immune system [Goldblum and Reed, 1980]. This is expressed by delayed or even no antibody production. It has been shown earlier that up to one-third of HCV PCR-positive patients on maintenance hemodialysis have no detectable antibody reactivity in different serological assays [Schröter et al., 1997], although usually simultaneous appearance of specific antibody reactivity and RT-PCR positivity can be observed in more than 98% [Feucht et al., 1997a]. The impaired immune system may also influence antibody production against GBV-C/HGV, resulting in an underestimate of the seroprevalence among chronic hemodialysis patients.

GBV-C/HGV viremia can be detected among hemodialysis patients in only 6%. This is three- to sixfold higher compared to the percentage of PCR positivity in voluntary blood donors [Jarvis et al., 1996; Linnen et al., 1996; Feucht et al., 1997b]. In contrast to the usual codetection of HCV antibodies and HCV viremia, in our setting GBV-C/HGV antibody reactivity and viremia were never detected at the same time. Indeed, parallel occurrence of GBV-C/HGV antibodies and viremia seems to be a rare event [Feucht et al., 1997b]. In

HCV-infected patients, antibodies against the NS3 region arise prior to those directed against other viral regions [Feucht et al., 1995]. Therefore, two recombinant proteins covering nearly the entire NS3 region of GBV-C/HGV have been included in the described immunoblot assay. Nevertheless, in the majority of serum samples seroreactivity is found without detectable viremia, which suggests that GBV-C/HGV infection might be self-limiting and does not become persistent [Feucht et al., 1997b; Thomas et al., 1998]. However, persistent GBV-C/HGV viremia below the detection limit of PCR cannot be excluded.

Like HCV, GBV-C/HGV can be transmitted parenterally [Linnen et al., 1996]. The three- to sixfold higher percentage of GBV-C/HGV PCR-positive samples among hemodialysis patients compared with voluntary blood donors has been interpreted as an indicator of increased risk for GBV-C/HGV transmission during hemodialysis [Masuko et al., 1996]. However, for HCV, which is known to be transmitted parenterally, a 30- to 40-fold higher prevalence among hemodialysis patients is observed compared with blood donors. Furthermore, in the general population HGV viremia is observed in 2%, HCV viremia only in 0.5% [Alter et al., 1992]. These observations indicate that significant, other than parenteral transmission routes must exist for GBV-C/HGV. It has been shown that GBV-C/HGV can be transmitted during pregnancy from mother to infant [Feucht et al., 1996]. Examination of homosexual men with no risk factors for parenterally transmitted diseases has revealed a high percentage of GBV-C/HGV infections, suggesting the possibility of sexual transmission [Stark et al., 1996]. This is supported by the observation that the GBV-C/HGV seroprevalence in the general population increases during the early life decades [Feucht et al., 1998].

The clinical impact of GBV-C/HGV is still controversial. In our study none of the GBV-C/HGV-seroreactive or GBV-C/HGV PCR-positive patients showed biochemical or clinical signs of hepatitis, except those who were also positive for HCV infection. Early reports stated that GBV-C/HGV might be responsible for acute, chronic, or fulminant hepatitis [Simons et al., 1995; Heringlake et al., 1996; Linnen et al., 1996]. Other groups found no correlation between the appearance of GBV-C/HGV and hepatitis [Wang et al., 1996; Alter et al., 1997]. The disease-inducing capacity of GBV-C/HGV in humans remains unclear.

The usage of separate dialysis machines for HCV-infected patients is a common measure taken to prevent nosocomial spread of HCV within hemodialysis units [Stuyver et al., 1996]. Although GBV-C/HGV viremia has been found in a high percentage among blood donors, general screening and exclusion of GBV-C/HGV-positive individuals from further donations have not yet been recommended [Alter et al., 1997]. For the same reason, at present we do not regard it as mandatory to dialyze GBV-C/HGV seroreactive or viremic patients on separate machines.

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